Development and early differentiation of male gonads in farmed New Zealand shortfin eel, *Anguilla australis*

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Abstract

This research provides the first description of the early gonad development and size at sexual differentiation for male shortfin eels in culture. Gonads from 7.5 – 10.0 cm eels were pear shaped containing two to six primordial germ cells. In 10.0 – 20.0 cm eels, gonads transition into a lamellar shape containing 5 – 50 primordial germ cells which multiply giving rise to clusters. Inter–sexual gonads showed the first signs of sexual differentiation, containing oogonia cysts, early meiotic ooyctes, clusters of primordial germ cells and occasional short rows of primary spermatogonia. Early testes were characterized by gonadal lamellae dominated by rows of primary spermatogonia with an early spermatic duct at its base. Eels with undifferentiated gonads were found across all size classes (7.5 – 30.0 cm), replaced by dominance of inter–sexual gonads in the 20.0 – 27.5 cm (55 %) and early testes in the 27.5 – 30.0 cm (60 %) size classes. Overall, male gonad differentiation for the New Zealand shortfin eel was found to progress in a similar manner to other Anguillid eels that have been studied under culture conditions.

Key words: *Anguilla australis*- gonad development - sexual differentiation - aquaculture.

Introduction

The shortfin eel *Anguilla australis*, Richardson 1849, is found throughout waterways of New Zealand, south-eastern Australia and the western Pacific Islands (Beumer 1996) and forms the basis of significant commercial, recreational, and Māori customary fisheries in New Zealand (McDowall 1990). Fishing effort combined with other anthropogenic factors, such as loss of habitat, migration barriers, and contamination of waterways has resulted in reductions of natural populations of shortfin eels, contributing to declining catches of adults during the

last 20 years (Ministry of Fisheries 2007).

Mature Anguillids are a gonochrostic species that begin development with an ambiguous primordial gonad (Colombo & Grandi 1996). This primordial gonad is undifferentiated and has low numbers of germ cells (Beullens *et al.* 1997). Males develop via a transitional intersexual stage where the gonads contains both spermatogonia and oogonia (termed a Syrski organ), whereas females develop ovaries directly from the undifferentiated primordial gonad (Colombo & Grandi 1996; Beullens *et al.* 1997).

Sex determination in *Anguilla* species is not unequivocally determined by genes, but is presumably influenced by environmental and social factors, with high temperatures and high densities biasing sex ratio towards males (Beullens *et al.* 1997; Krueger & Oliveira 1999). Anguillids also show clear sexual dimorphism (Krueger & Oliveira 1999), with males growing faster than females but females attaining greater body size than males (Holmgren *et al.* 1997).

Despite an incomplete understanding of the factors controlling sex determination in eels, a number of methods are available to manipulate sex ratios in cultured populations. These are classified as direct intervention methods, whereby synthetic or natural hormones are applied to artificially stimulate sexual development, and indirect approaches, which promote the development of one sex over the other by altering environmental conditions (Davey & Jellyman 2005). The possibility that the gender of eels can be manipulated provides potential commercial and ecological benefit. First, females reach a higher mass and market value, but the sex ratio is skewed toward males in eel stocks under intensive culture conditions (Colombo & Grandi 1996; Holmgren 1996; Beullens *et al.* 1997). The ability to control sex expression and rear more females could therefore greatly increase eel production and produce significant economic benefits (Grandi *et al.* 2000). Second, fisheries for wild eels preferentially harvest females because of their greater size and longer duration in fresh water (Beentjes 1999). This selective harvest has given rise to concerns about the well-being of stocks of some species, including the New Zealand shortfin eel *A. australis* and the New Zealand longfin eel, *A. dieffenbachii* (Ministry of Fisheries 2007). Sex manipulation offers one approach to conserving and sustainably managing wild populations (Davey & Jellyman 2005).

The difficulty of distinguishing testes and ovaries during the early stages of gonad development means that the exact timing of sexual differentiation is sometimes uncertain (Tesch 1977). Consequently, many researchers studying sex ratios and gonadal development now depend on histological examination for accuracy, particularly with small immature individuals. While there have been investigations of gonad morphology and development in Anguillid species, the studies dealing with reproductive development in shortfin eels have focused on changes associated with maturation (Todd 1974; Todd1980; Lokman *et al.* 1998a, 1998b).

The aim of the present study was to use microscopic (histological) techniques to determine the size at which the first stages of gonad sexual differentiation occur in male shortfin eels reared under experimental culturing conditions in 23 °C brackish water (15-20‰). These culture conditions were selected because this experiment was part of a larger project investigating the feasibility of culturing shortfin eels in brackish water. It is generally accepted that eel gonad develop-

ment correlates from the earliest stages with body size (total length) rather than with age (Oliveira & McCleave 2000), therefore different stages of gonad development were evaluated against total body length to determine the size at which the first stages of sexual differentiation.

Methods

Glass Eel Collection and Culture

Shortfin glass eels were collected from the Waikato River (North Island New Zealand) using modified dip nets on 23 September 2003 and were transported in 45 l insulated 90 plastic bins, containing 20 l of 13° C fresh water to the NIWA Bream Bay Aquaculture Park, north–eastern New Zealand. On arrival a random sample of 100 glass eels was taken and then each eel was assessed for stage of pigmentation based upon standards developed by Strubberg (1913) with levels ranging between stages 5b and 6a23.

Glass eels were reared for 456 d in a commercially produced recirculation system (Hesy) designed specifically for eel aquaculture. The eels were reared in four replicate 8500 l white circular plastic tanks filled with 7000 l of filtered (7 µm) brackish water (17.5 ‰) maintained by heat pump at 23 ± 3.5° C, with a water flow of approximately 3 l min^{-1}. The dissolved oxygen level was maintained at > 4.5 mg l^{-1} and pH was maintained between 7.0 and 9.0. The tanks were cleaned daily, removing any dead animals and food residues. Approximately 10% of water was renewed daily. Eels were fed a commercial pellet diet (DANEX) at a rate of 5% body weight day^{-1} delivered continuously over 24 h on an automated band feeder. The biomass of eels from each tank was weighed every 14 d to calculate feeding rate. Large variations in size are common in eels of the same age reared under identical culture conditions. Throughout the experiment (approximately 42 d intervals) eels were graded and allocated into one of the four rearing tanks according to body size, providing a more homogenous spread of eel size within each tank.

Gonad Histology

Body length was categorised into nine 2.5 cm size classes, the first size class ranging from 7.5 – 10.0 cm, the last from 27.5 – 30.0 cm. Twenty eels from each successive size class were randomly collected and anaesthetized with AQUI-S®, individually measured for total length and weight, and killed by decapitation. A 10 mm wide section was removed from the central gonadic region (mid-way between the pectoral fin and anal opening) and fixed in 10 % buffered formalin solution. The sample tissue was processed in a Tissue Tek V.I.P. 2000 Vacuum Infiltration Processor, dehydrated with ethanol, cleared with xylol, infiltrated and embedded into paraffin (paraplast 56 °mp.) wax blocks. Using a microtome, 7 µm thick sections were cut from three regions within the gonad area, mounted onto poly–L–lysine treated slides and stained with haematoxylin (Gill's II Haematoxylin) and eosin (1% EosinY, aqueous). Slides were examined under a compound microscope. Based on a series of criteria specific to the genus *Anguilla* (Beullens *et al.* 1997; Walsh *et al.* 2003), gonads were classified as undifferentiated, inter–sexual or early differentiated testes. In sections where more than one stage was apparent, the sample was categorized according to the most dominant cell type present. For gonadal tissue where the sex could not be determined, an undifferentiated stage was designated. Germ cell diameter was determined by measuring five of the most dominant cells from each section using a

micrometer eyepiece. Images of gonad samples representative of each development stage were recorded using a digital camera mounted on the eye piece of a compound microscope.

Statistical Analyses

Differences in the mean length of eels at different stages of gonadal developmental were identified using a one–way ANOVA followed by a post–hoc Tukey's LSD multiple comparison test. Data were considered significant at $P \leq 0.05$. All mean values are presented as mean ± SE.

Results

Histological Observations

7.5 – 10.0 cm eels

The gonads of 7.5 – 10.0 cm eels is pear shaped and enclosed by a monolayered peritoneal epithelium with flattened cells on the vascular side and thicker cells on the germinal side and attached to the dorsal wall of the abdominal cavity on both sides of the mesentry by a relatively long mesogonadium. Gonads contained between two to six primordial germ cells per cross section. The primordial germ cells were large ovoid cells, 10 – 12 µm in size, with an extensive cytoplasm and large distinct central nucleus, 5 – 6 µm in diameter. Each primordial germ cell was enveloped by four or five flattened somatic cells (Figure 1).

10.0 – 12.5 & 12.5 – 15.0 cm eels

In the 10.0 – 12.5 and 12.5 – 15.0 cm size class, the peritoneal epithelium encasing the gonads was composed of flattened cells on the medial and distal side, and of thicker cells on the proximal and lateral side. The mesogonadium had thickened and was characterized by connective tissue rich in bundles of collagen fibrils, fibroblasts and capillaries. Laterally, the lamellae had an irregular outline and were formed of larger cells, some of which were inserted between the germ cells facing the lamellar border. At 10.0 – 12.5 and 12.5 – 15.0 cm of length, the gonads were transitioning from a pear shape to a lamellar blade shape. Gonads contained three to 12 primordial germ cells per cross section. Overall, histological features were similar to those of the 7.5 – 10.0 cm eels, with gonadal lamellae size and primordial germ cell number increasing with eel length. Primordial germ cell diameter increased to 20 µm with a 6 – 7 µm nucleus (Figure 2).

15.0 – 17.5 cm eels

The gonadal lamellae of eels of 15.0 – 17.5 cm in length were larger than the earlier size class and contained considerably more primordial germ cells (15 – 50) per cross section. The gonadal lamellae had distinct vascular and germinal regions located on the medial and lateral sides respectively. In the vascular region, the stromal tissue was abundant with large bundles of collagen fibrils, several fibroblasts, and blood capillaries. The germinal region consisted of primordial germ cell complexes and clusters of germ cells enveloped by flattened somatic cells. The majority of clusters were formed of three or four germ cells in contact with each other or partially separated by flattened somatic cells, a configuration consistent with their development by introgonadic mitosis of primordial germ cells. The clustered primordial germ cells are round, 6 – 8 µm in diameter, with cytoplasm slightly darker than that of single primordial germ cells and a circular nucleus of 4 – 5 µm in diameter with an evident nucleous (Figure 3).

17.5 – 20.0 cm eels

In eels 17.5 – 20.0 cm in length the gonadal lamellae were more elongated than those of the previous size class containing 40 – 50 germ cells per cross section. The germinal region consisted of primordial germ cells and clusters of primordial germ cells surrounded by numerous dark polymorphic somatic cells in an abundant compact connective tissue.

20.0 – 22.5 & 22.5 – 25.0 cm eels

In the 20.0 – 22.5 and 22.5 – 25.0 cm size class, lamellar gonads were found with three kinds of histological structure. One type had a structure similar to that of the 17.5 – 20.0 cm eels and was classified as undifferentiated.

In the second type, the germinal region of the gonads contained cysts of oogonia and of synchronous early meiotic ooyctes in addition to primordial germ cells and clusters of primordial germ cells surrounded by numerous dark polymorphic somatic cells in an abundant compact connective tissue. The cysts were enveloped by a single layer of flattened, dark somatic cells and oogonia were characterised by a round cell body of 6 – 8 µm in diameter, with cytoplasm lighter and nucleus darker than clustered primordial germ cells. The ooycte was 10 – 11 µm in diameter, with light cytoplasm and light round nuclei showing chromosomal threads (bivalents). Occasional short rows of primary spermatogonia similar in size as clustered primordial germ cells but darker and surrounded by larger polymorphic dark somatic cells (pre–Sertoli) were occasionally observed (Figure 4). Gonads of this type were classified as inter–sexual (Syrski organs), according to terminology proposed for the European eel by Columbo & Grandi (1996).

A third type of gonad was characterized by a large gonadal lamellae dominated by rows of primary spermatogonia and connected to the abdominal walls by a large mesorchium with an early spermatic duct at its base. This type was considered to be a transition from an inter-sexual gonad to a more advanced stage of early testes.

25.0 – 27.5 & 27.5 – 30.0 cm eels

Undifferentiated, inter-sexual and early testes gonads were observed in the 25.0 – 27.5 and 27.5 – 30.0 cm size classes, with noticeable advancement of gonad development towards early testes.

Eel size at sexual differentiation

Eels with undifferentiated gonads were found across all size classes (7.5 – 30.0 cm), replaced by dominance of inter–sexual gonads in the 20.0 – 27.5 cm (55%) and early testes in the 27.5 – 30.0 cm (60%) size classes (Figure 5). The first sign of sexual differentiation, recognisable through the appearance of oogonial cysts, was observed in an eel of 20.0 cm possessing an inter–sexual gonad. The mean length of eels with inter–sexual gonads (23.9 ± 0.3 cm) appeared to provide a reasonable estimate of size at differentiation. There was an overall absence of ovarian differentiating gonads in the present study. Mean eel length was significantly different (ANOVA, $F = 163.27$, $P < 0.05$) among gonadal development stages (Figure 6). A post–hoc Tukey's LSD multiple comparison test showed that mean eel length increased significantly with each successive gonadal development stage, i.e., undifferentiated, inter–sexual and early testes ($P < 0.05$). However, there was a high degree of overlap in body size between each successive gonadal development stage.

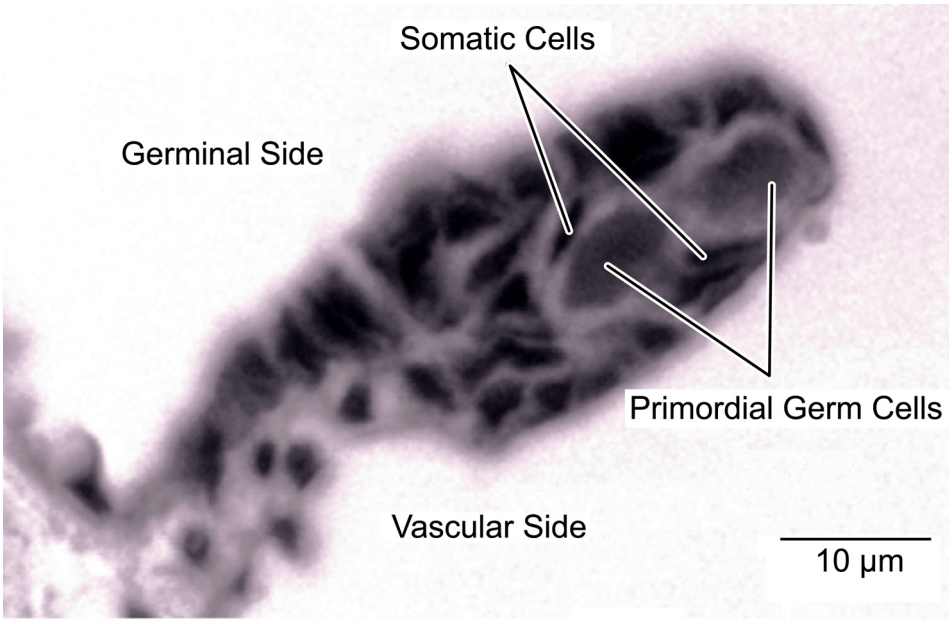

Figure 1. Transverse section of a gonad from an 8.9 cm eel containing two primordial germ cells enveloped by somatic cells.

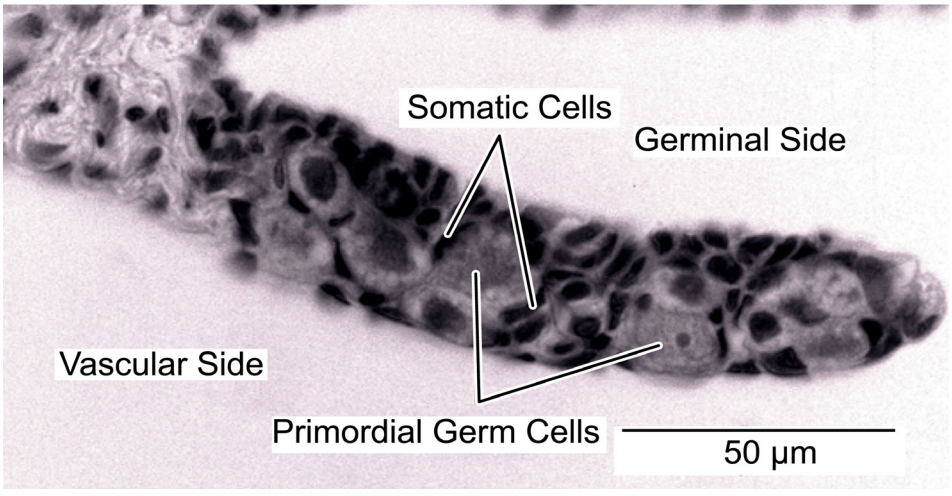

Figure 2. Transverse section of a gonad from an 11.2 cm eel containing primordial germ cells. Note the gonads were transitioning from a pear shape to a lamellar blade shape.

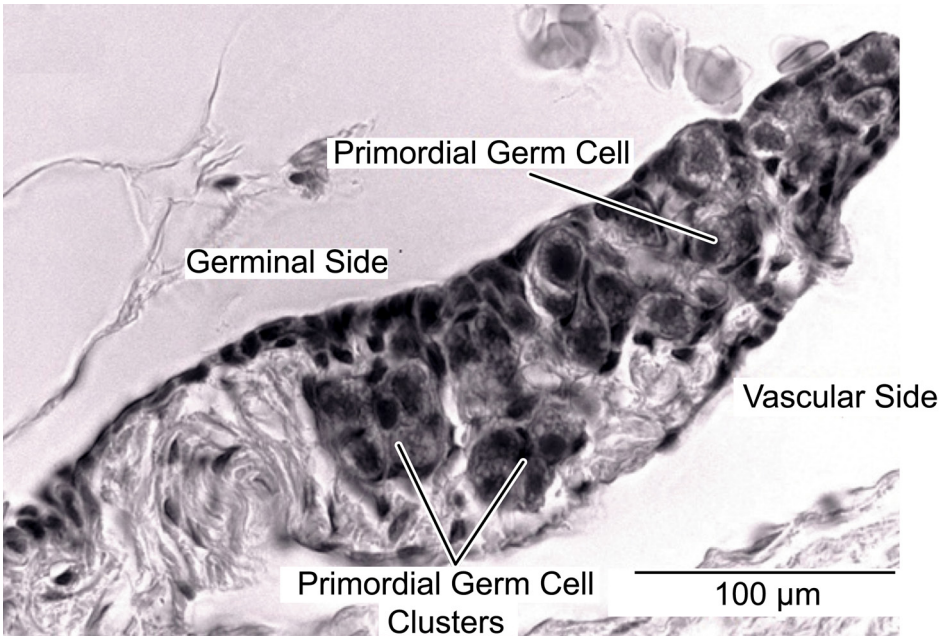

Figure 3. Transverse section of a gonad from a 17.0 cm eel containing primordial germ cells and clusters of secondary primordial germ cells. Note the lamellar blade shape of the gonad.

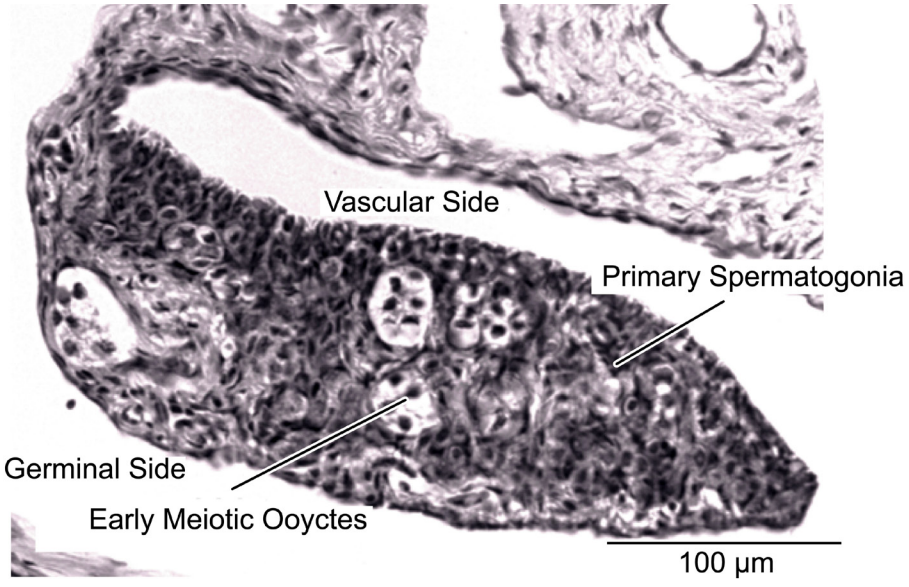

Figure 4. Gonadal lamella from a 23.3 cm eel, exhibiting the structure of an inter–sexual gonad. Note the appearance of early meiotic ooyctes and primary spermatogonia.

Discussion

This research provides the first description of the early gonad development and size at sexual differentiation for male shortfin eels in culture, providing a benchmark on which to base future experiments aimed at attempting to manipulate sex determination by varying culture conditions or hormonal intervention. Under the culture conditions used in this study the size range at which shortfin eel gonads first sexually differentiated to males was 20.0 – 22.5 cm long. The first sign of sexual differentiation, recognisable through the appearance of oogonial cysts, was observed in an eel of 20.0 cm length. As shortfin eel length increased from 20.0 cm, the frequency of undifferentiated gonads declined, with inter-sexual gonads becoming dominant in the 20.0 - 27.5 cm size class, and early testes dominant in the 27.5 – 30.0 cm range.

Studies on other Anguillid species indicate gonad differentiation rarely commences at lengths less than 15.0 cm, but shows significant variation among species. In the European eel, *A. anguilla,* and the American eel, *A. rostrata*, sexual differentiation commences around 20.0 cm in length and is often complete at 35.0 cm (Colombo *et al.* 1984; Amin 1997; Beullens *et al.* 1997; Krueger & Oliveira 1999; Oliveira & McCleave 2000). However, the New Zealand longfin eel, *A. dieffenbachii*, and the Australian longfin eel, *A. reinhardtii*, in which both sexes attain larger maximum sizes, are often sexually indeterminate until they reach 45.0 cm in length (Harries 1974; Walsh *et al.* 2003).

The development of shortfin eel gonads follows similar stages as observed in other Anguillid species (Satoh *et al.* 1962; Tesch 1977; Beullens *et al.* 1997;

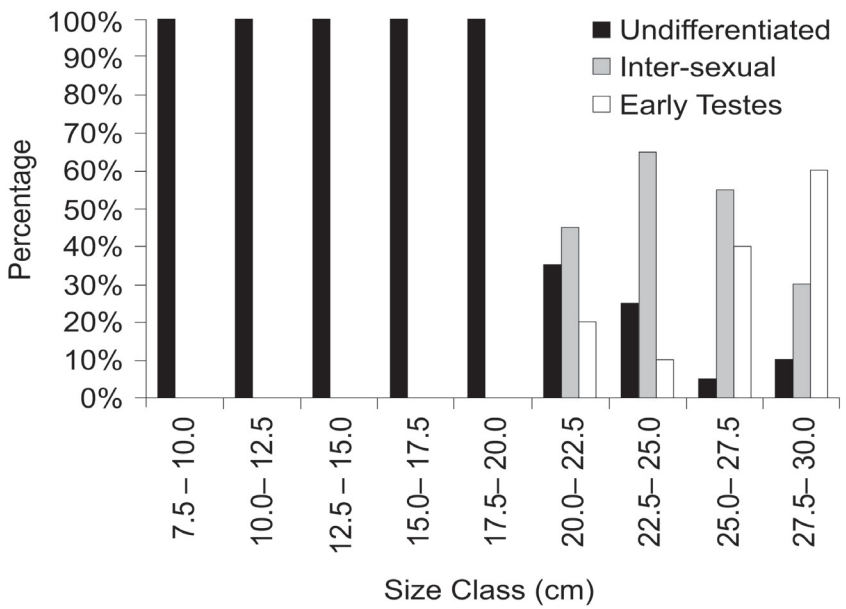

Figure 5. Length frequency distribution of shortfin eels at various stages of gonadal development (n = 20 per size class).

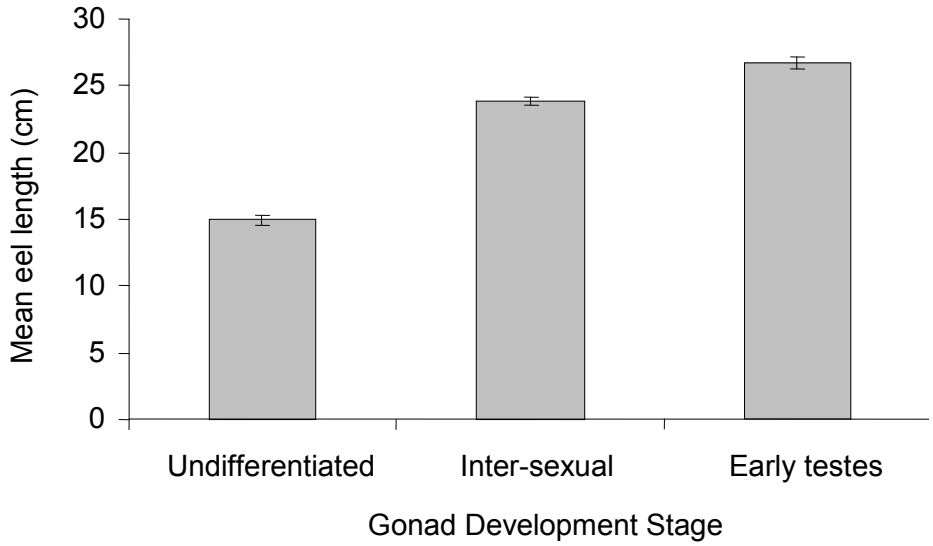

Figure 6. Mean (± SE) length of shortfin eels at various stages of gonadal development (undifferentiated $n = 106$, inter–sexual $n = 40$, early testes $n = 34$). All pairwise mean comparisons were significantly different at $P > 0.05$.

Colombo & Grandi 1996; Krueger & Oliveira 1999). As eels increase in size (7.5 – 15.0 cm) the gonads enlarge and transition from a pear to lamellar shape and the number of primordial germ cells increase. In 15.0 – 20.0 cm eels some primordial germ cells multiply, giving rise to clusters of primordial germ cells and the lamellae increases in size. The gonads of eels < 20.0 cm were classified as undifferentiated, showing no histological sign of sex differentiation.

For eels 20.0 – 30.0 cm the gonad further increased in size maintaining the lamellae shape and exhibiting rows of primordial germ cells and clusters of primordial germ cells (both sexually undifferentiated germ cells). The emergence of oogonia cysts and oocytes (female germ cells), which are the first cells in eels to differentiate sexually, mark the start of gonad differentiation (Colombo & Grandi 1996). Occasional short rows of primary spermatogonia, which were about the same size as clustered primordial germ cells, but darker and surrounded by larger polymorphic dark somatic cells (pre–Sertoli) were observed. The primary spermatogonia were individually enveloped by somatic cells. Gonads with the above histological features correspond to small testes, containing both male and female germ cells, which we define as inter–sexual gonads (Syrski organs). This inter–sexual characteristic is also documented in the European (Sinha & Jones 1966; Beullens *et al.* 1997; Grandi & Colombo 1997) and American (Helfman *et al.* 1987) eel species.

In eels greater than 20.0 cm in length the early testes were observed to differentiate from the existing inter–sexual gonad which was present in all of the 100 eels of a smaller size that were examined. These developing gonads were characterized by a more advanced stage towards early testes because they appeared as large gonadal lamellae dominated by rows of primary spermatogonia and connected to the abdominal walls by a large mesorchium with

an early spermatic duct at its base. At the same time the oocytes, previously present in the inter-sexual gonad, degenerate and disappear. Early ovaries are characterized by lamellae containing double rows of previtellogenic oocytes at the perinucleolus stage, running from the vascular to the germinal region by a thin mesogonadium (Grandi & Colombo 1997).

A long held view is that sex is determined principally by eel density, with low densities tending to favour development of eels as females, while high densities favour the development of males (Colombo & Grandi 1996; Holmgren 1996; Beullens *et al.* 1997). The stocking density of fish in the current trial was not recorded, yet would be considered high when compared to wild fisheries and may provide an explanation for the absence of any experimental eels differentiating into females. However, the maximum size of eels sampled (30.0 cm) in the current experiment may have been below the minimum threshold required to observe ovarian differentiation, as Todd (1974) showed that wild shortfin females do not differentiate until 32 cm. Further research raising eels to larger size classes would reveal the presence and timing of any female sexual differentiation.

The high proportion of shortfin eels differentiating as males is a significant marketing constraint on the aquaculture production of this species because male eels grow to a smaller maximum size than females. There are a number of methods available to manipulate sex ratios in cultured populations of eels. Natural and synthetic sex steroids can have a strong feminizing effect on undifferentiated individuals, especially when administered at high doses for prolonged periods to young eels (Davey & Jellyman 2005). Reducing eel density and limiting interference and social stress may also promote the development of females in captive populations (Beullens *et al.* 1997; Krueger & Oliveira 1999). However, the proximate and ultimate factors influencing sex determination and differentiation in eels remain poorly understood despite an abundance of descriptive information. Further research into the timing and mechanisms of sex determination in shortfin eels is now required to effectively and efficiently manipulate sex for improving the potential for aquaculture production from this species.

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